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GST Gene Variants in Synchronous Colorectal Cancers and Synchronous Association of Colorectal Cancers with Other Cancers

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Rezumat

Variante genetice de glutation-S-transferază incriminate în formele sincrone de cancer colorectal şi în asocierile sincrone ale acestuia cu alte tipuri de cancer

Obiectiv: studiul de faţă evaluează polimorfismele genetice a trei glutation S-transferaze (GSTM1, GSTT1 şi GSTP1) la pacienţii cu tumori maligne multiple colorectale şi asociere de cancer colorectal cu alt cancer.
Material şi Metodă: din cadrul a 420 pacienţi diagnosticaţi în spitalul nostru cu cancer colorectal în perioada 2005-2012, am selectat pentru analiză genetică 20 pacienţi cu cancere sincrone colorectale şi 9 pacienţi cu asocierea unui cancer colorectal cu un alt cancer, toţi rasă caucaziană. Am căutat genotipurile GST, comparându-le cu cele existente la un lot de martori.
Rezultate: analizele genetice au fost posibile doar pentru 19 pacienţi cu cancere sincrone colorectale şi 9 pacienţi cu asociere de cancer colorectal sincron cu un alt cancer; am găsit prezenţa unei diferenţe statistice semnificative, în comparaţie cu lotul martorilor, a frecvenţei genotipului nul GSTM1; nu am găsit diferenţe ale frecvenţei genotipului nul GSTT1 şi a polimorfismului Ile105Val al GSTP1 la aceşti pacienţi.
Concluzie: în studiul nostru genotipul nul GSTM1 este un factor de risc pentru cancerele sincrone colorectale şi pentru asocierea sincronă a unui cancer colorectal cu un alt cancer.

Cuvinte cheie: cancere colorectale, leziuni sincrone, alte cancere, GSTM1, GSTT1, GSTP1.

Abstract

Background: the present study evaluates genetic polymorphisms of three glutathione S-transferases (GSTM1, GSTT1 and GSTP1) in patients with synchronous malignant colorectal tumors and the association of synchronous colorectal cancers with other cancers.
Material and Methods: from 420 patients with a colorectal cancer admitted to our hospital between 2005-2012, we selected for genetic analysis 20 patients with multiple synchronous malignant colorectal tumors and 9 patients with a synchronous association of colorectal cancer with another cancer. We searched for GST genotypes, comparing the results with controls.
Results: the genetic analysis was possible only in 19 patients with colorectal synchronous cancers and 9 patients with a synchronous association of colorectal cancer with another cancer; we found a statistically significant difference for null

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GSTM1 genotype frequency between these patients and the control group; we found no differences regarding the frequency of null GSTT1 genotype and Ile105Val polymorphism of GSTP1 in patients with synchronous cancers compared with the control group.
Conclusion: in our study we found the null GSTM1 genotype as a risk factor for multiple colorectal synchronous cancers and for an association of synchronous colorectal with other cancers.

Key words: colorectal cancers, synchronous lesions, other cancers, GSTM1, GSTT1, GSTP1

Introduction

In 2008, colorectal cancer was the third most common cancer in men and the second most common one in women all over the world (1). Synchronous malignant colorectal tumors represent only 4-6% of neoplasms (2-7). Glutathione S-transferases (GST-ies) are a major group of detoxification enzymes, present in all eukaryotic species and in humans, involved in the metabolism of a large variety of xenobiotic compounds, catalysing the conjugation of various electrophilic compounds with glutathione, giving rise in most cases to less reactive, water-soluble metabolites that are more easily excreted through urine. These family members have many other functions: regulation of transcription and translation (8), intracellular transport of ions (9,10). In humans, four classes of cytosolic GST isoenzymes are identified (alpha, mu, pi, and theta), many of them being polymorphic.

Material and Methods

Between January 2005 and June 2012 in Cluj-Napoca 5th Surgical Clinic 420 patients were diagnosed and treated for colorectal cancer. TTwenty-nine patients (6.90%) were diagnosed with synchronous multiple cancers: 20 (4.76%) with synchronous colorectal cancers and 9 (2.14%) with synchronous association of a colorectal cancer and another cancer. We considered synchronous cancers those appeared within 6 months after the first one diagnosed. Eighteen patients underwent surgical procedures, one had tumor biopsies through colonoscopy and diagnosis of malignant tumors but never came in for surgical treatment and in one a chemotherapy protocol was initiated because of an advanced disease. The associations of synchronous cancers and surgical therapy were earlier described (11). There were 13 males with an average age of 63.15 years (range 39 to 76 years) and 7 females with average age of 71.28 years (range 55 to 81 years). Together they had 53 malignant tumors, 25 of them in the right colon, 17 in the left colon and 11 in the rectum. 14 patients are still alive, even if some of them have an advanced disease with liver metastases. Apart from colorectal cancers, the synchronous cancers from the other 9 patients (6 males and 3 females) associated kidney (5 cases), prostate, gastric, pancreatic and breast cancer (in one case each). One patient had a skin cancer one year earlier. The colorectal cancer involved the rectum (6 patients), descending colon (2 patients) and the transverse colon in one. The average age for the whole group was 67.66 years. Presently 8 patients are alive.

All these patients and a corresponding number of controls (matched for age and sex) were analysed for GSTM1, GSTT1 and the Ile105Val polymorphism of GSTP1 gene. The study was approved by the Clinic County Hospital Ethics Board. The patients and controls have signed an informed consent for genetic research; for the patients who died before beginning the DNA extraction, the informed consents preoperatively signed were used (which allow using data and biological material, under protection of anonymity). We used 2 ml of peripheral blood and paraffin embedded samples from biopsies for DNA extraction.

In one patient the DNA was too fragmented (the DNA was extracted from a paraffin-embedded tumor) and it was not possible to read the results. The presence or absence of the GSTT1 or GSTM1 genes was simultaneously determined using a multiplex PCR protocol previously described (12) being able to identify only the null genotypes, but not being able to detect heterozygous (+/0) genotypes. We used 3 primer pairs for amplification of 215 bp in case of GSTM1 allele and 480 bp for GSTT1 allele (FwM1 5'-GAACTC CCTGAAAAGCTAAAGC-3'; RevM1 5'- GTTGGGCT-CAAATATAGGGTGG- 3'and FwT1 5'-TTCCTTACT GGTCCTCACATCTC-3'; RevT1 5'- TCACCGGAT-CATGGC CAGCA-3'). As an internal amplification ccontrol, the primer pair for a co-amplification of 268 bp of β Globin gene was used. We accomplished the PCR reactions in a gradient thermocycler (Mastercycler Gradient, Eppendorf®, Germany). 100 ng of genomic DNA were amplified in a final volume of 25 μl reaction mixture containing 12.5μl 2xPCR Master Mix (Fermentas MBI, Lituania®), 1μl BSA (Bovine Serum Albumine, Fermentas MBI, Lituania®) solution 5 mg/ml, 8 pM of each primer, forward and reverse (Eurogentec, Belgium®) and DNase/RNase free water to complete the 25 μl reaction volume. We analysed the amplification products using electrophoresis in MetaPhor agarose gel (Lonza®, Basel, Switzerland). The null genotypes were considered in the absence of amplification products (215 or 480 bp).

For the detection of GSTP1 polymorphism we used a Wizard Genomic DNA Purification Kit (Promega®, MA, USA) in order to extract genomic DNA from 300μl of blood (leucocytes). The Ile105Val polymorphism of GSTP1 gene was analysed by the polymerase chain reaction (PCR) - restriction fragment length polymorphism (RFLP) technique as described previously (13), with some modifications. We used for the amplification of the DNA the primer pair 105F (5'-ACCCCAGGGCTCTATGGGAA-3') and 105R (5'-TGAGGGCACAAGAAGCCCCT-3'), 12.5μl 2 x PCR Master Mix (Fermentas MBI, Lituania®), 1μl BSA (Bovine Serum Albumine, Fermentas MBI, Lituania ®) solution 2

mg/ml, 8 pM of each primer, forward and reverse (Eurogentec, Belgium®) and water free of nucleases to complete the 25 μl volume. The PCR reactions were done using a gradient thermocycler (Mastercycler Gradient, Eppendorf®). The 176 bp PCR product was digested with 5 U BsmAI (Fermentas MBI, Lituania®), and the fragments were separated on a 3.0% Metaphor® agarose gel (Lonza®, Basel, Switzerland), and visualized in a UV transilluminator (VilberLourmat Imaging System®, Marne-la-Vallée, France) after staining with ethidium bromide. After digestion three fragments of 176.91 and 85 bp were obtained, for the Ile/Val genotype. Two fragments of 91 and 85 bp, characterise the Val/Val homozygous genotype. The uncut product (176 bp) corresponds to the absence of a restriction site, and Ile/Ile genotype (14).

The DNA extraction from paraffin embedded samples consisted in a multiple step procedure, and was based on a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), according to manufacturer instructions.

Statistical analysis was performed using Medcalc v12.3. Deviations of allelic frequencies from Hardy–Weinberg equilibrium were computed using a chi-square test. Descriptive analysis included frequencies for nominal variables and the median for continuous variables. The chi-square test was used to compare the frequency of nominal variables and for computing odds ratio. The level of statistical significance was set at $p<0.05$.

Results

Genotyping was performed for 28 patients. For the synchronous colorectal cancers group we had 19 patients, along with 19 healthy controls of the same age (±2 years). The average age of the patients at diagnosis with synchronous colorectal cancers was 65.87±11.05 years (range 39 to 81 years). The average age of the control group was 64.53±10.45 years (range 37 to 79 years). We had 13 women (34.2%) and 25 men (65.8%). Fifteen patients (78.9%) from the multiple cancers group and 5 from the control (26.3%) were smokers, the percentage of smokers in the oncological group being statistically greater ($p=0.003$). We did not observe differences of age ($p=0.1$), sex ($p=0.1$) or cancer location ($p>0.05$) between smokers and non-smokers. The GSTM1 null allele was found in 14 oncological patients (73.6%), meanwhile in the control group it was found only in 4 subjects (21%); we found a Hardy-Weinberg equilibrium for the alleles and statistically significant differences of the null allele in the oncological group ($p=0.003$). The GSTT1 null genotype was found in 4 patients (21%) and in 3 controls (15.7%), the alleles being in a Hardy-Weinberg equilibrium, with no statistically significant differences of the frequency of null genotype in patients comparing with controls ($p=1$). Four patients (21%) from the patient series had Ile105Ile genotype of GSTP1, 8 patients (42.1%) had Ile/Val genotype (heterozygous) and 3 patients (15.7%) were homozygous for variant allele (they had the GSTP1 polymorphism Val105Val). In the control group 9 (47.3%) were homozygous for the common genotype (Ile/Ile), 8 (42.1%) were heterozygous Ile/Val and 2 (10.5%) were homozygous variant alleles (the polymorphism Val/Val). The allele frequency did not deviate from the Hardy-Weinberg equilibrium. There were no statistically significant differences between the variant allele in the patient and control groups ($p=0.4$). We did not find statistically significant differences of both GSTM1 and GSTT1 null alleles for the different sites of the malignant tumors ($p>0.05$). The GSTT1 null genotype had no statistically significant differences for the different localisations of the colorectal cancers ($p>0.05$). The frequency of the GSTP1 Ile105Val polymorphism was not different between different sites of the colorectal malignant tumors ($p>0.05$). Both null GSTM1 and GSTT1 genotypes were present at the same time in 2 patients (10.5%) and 3 controls (15.7%), but the difference was not statistically significant ($p=1$). There were 16 patients (84.2%) and 4 controls (21%) with at least one null genotype either for GSTM1 or GSTT1. The difference was highly statistically significant (χ^2 test; $p<0.001$). The odds ratio (OR) for a GSTM1 null genotype for developing synchronous colorectal cancers was 3.1 (CI 95%, 1.4-6.9), and in case of any null allele (GSTM1 or GSTT1) the OR was 4.8(CI 95%, 1.6-13.8).

The other 9 patients with an association of synchronous colorectal cancer and another cancer were compared with 9 healthy control subjects, of ±2 years. The average age of the patients was 67.67±8 years (56 to 77 years). The average age for controls was 68±8.1 years (56 to 78 years). Together, there were 6 women (33.3%) and 12 men (66.7%) 5 patients (55.5%) and 6 controls (66.7%) were smokers, without statistically significant differences between the two groups ($p=1$). The GSTM1 null genotype was found in 8 patients (88.8%) and 3 controls (33.3%). For the association of synchronous colorectal cancers with other types of cancer OR=5 for a null GSTM1 genotype. The GSTT1 null genotype was found in 2 patients (22.2%) and 1 control (11.1%). There were no statistically significant differences between oncological patients and controls ($p=1$). The GSTP1 homozygous Ile/Ile genotype was found in one patient (11.1%) and 3 controls (33.3%), the heterozygous Ile/Val genotype in 5 patients (55.5%) and 5 controls (55.5%), the homozygous Val/Val genotype in 2 patients (22.2%) and 1 control (11.1%). There were no statistically significant differences between patients and controls for Ile105Val polymorphism ($p=0.5$). The risk (OR) for synchronous association of colorectal cancer with another cancer linked with null GSTM1 genotype was 5 (CI de 95%, 0.8-32.2).

Discussions

In the United States a continued decline in incidence and in mortality rates for almost all cancers combined both for men and women, greater for men than for women was documented, but it was influenced by specific types of cancer, sex, racial or ethnic groups (15). Even if global incidence rates in the United States have decreased, an increasing incidence rate among men and women under 50 years of age was observed, knowing that screening programs concern people

over 50 years (16). In 2008, in Europe, colorectal cancer occupied the third place among the most frequent cancers in men, after prostate and pulmonary cancer and in women it occupied the second place, after breast cancer; for both sexes the first place was held by colorectal cancer (17). Romania was in 2008 in a middle position for both the incidence and the mortality rate for both sexes: in men the incidence rate was 27.6 (4554 cases), with a mortality rate of 16.7 (2884 deaths); in women the incidence rate was 19 (4142 cases), with a mortality rate of 9.7 (2294 deaths). The mortality after colorectal cancer continues to decrease, this reflecting a decrease of its incidence and an improvement of survival (18). In colorectal cancers an improvement of prognosis was observed, mainly due to an earlier diagnosis thanks to imagistic methods and also because of surveillance and screening programs for diseases known to have a predisposition or an increased risk for development of colorectal cancers.

Lynch syndromes, hereditary diseases determined by mutations in the DNA mismatch-repair genes, are responsible for 2-4% of all colorectal cancers (19) and for 2-5% of all endometrial cancers (20). In our series there were no Lynch syndrome patients with multiple cancers. In the general population the lifetime risk to develop a colorectal cancer is 2% (21). Cunliffe (22) cited von Czerny who described in 1880 multiple primary adenocarcinomas of the large bowel. Synchronous colorectal cancers are defined as tumors diagnosed either preoperatively, during an operation by palpation, or postoperatively by colonoscopy within a period less than 6 months; the lesions have to be at least 4 cm distant from each other; the tumors should not consist of submucosal spread or as a satellite lesion of each other (22). A metachronous cancer is when the second tumor is diagnosed more than 6 months after the first tumor (7, 23), which is called the index tumor. It is important to distinguish between a recurrence and a new primary cancer. Multiple primary colorectal cancers may be diagnosed if a simultaneous primary lesion is overlooked, or if a metachronous second primary cancer develops, or when a malignant transformation in a neighbouring polyp appears (23).

Multiple synchronous colorectal cancers are found more frequently after skin and breast cancer (23). In our series of synchronous cancers we had: basocellular carcinoma of the skin, pancreatic, breast, gastric, prostate and especially kidney cancer. The reported percentage of synchronous primary colorectal cancers was between 2.8-6.7% (24-32). In comparison with the general population, synchronous cancers appear more frequently in patients with ulcerative colitis (34, 35) or familial adenomatous polyposis (36). A localisation on the right colon was observed for the second primary colorectal cancer in a series of 2524 patients surgically treated (37). In our patients, almost half presented with tumours localized on the right side of the colon.

Between January 2005 and June 2012 we had 420 cases of colorectal malignant tumors (in this number we did not include the patients with a history of colorectal cancer), admitted and surgically treated, and among them 29 had multiple synchronous cancers (6.90%), not only with a colorectal localisation. There were only 5 cases of metachronous colorectal lesions. Also, in our series, three patients developed metachronous malignant tumors and some of them were synchronously discovered. The survival rate was 70% (14 patients from 20) for synchronous colorectal cancers and 88.88% (8 out of 9 patients) for synchronous cancers of the colorectum and another type of cancer. It is considered that the most frequent sites for an extracolonic primary cancer are: the skin, stomach, breast, urinary bladder, and prostate (38-40) and that cancers may occur between 17 years before and 20 years after the diagnosis of the colorectal cancer (41,42). In our series, the kidney was the most common site for another cancer, discovered synchronously with the colorectal cancer; also, there were breast, pancreatic, gastric, prostate and skin (non-melanoma) cancers.

The theta class (43) of GST includes GSTT1 (44) and GSTT2 (45) two proteins which share 55% amino acid sequence identity (46) and which are both involved in human carcinogenesis (47) GSTT1 is located on chromosome 22q11.2 (48) while GSTM1 is located on chromosome 1p13.3. The GSTT1 gene is absent from 38% of the population and 16% of Europeans have a GSTT1 null genotype (49). GSTM1 is present in 55% of the population (50). 40-60% of the Caucasian population has a homozygous deletion of the GSTM1 gene or the GSTT1 gene (51), which determines a complete loss of these isoenzymes having detoxifying roles. Null mutations of GSTM1 gene and GSTT1 gene have been linked with an increase in a number of cancers (colorectal cancers as well), probably due to an increased susceptibility to environmental toxins and carcinogens (52-56). The glutathione is a major antioxidant used by GST to neutralize reactive oxygen products and xenobiotics, protecting the cells from damage due to environmental toxins and carcinogens. Because of GSTs all these toxic agents become water-soluble and may be excreted from the body (57). It is considered that people carrying the null genotypes of GST may have higher levels of intermediates of oxidative metabolism (highly reacted oxygen products) because the detoxification pathways are disrupted.

The GSTP1 gene is located on the long arm of chromosome 11 (11q13.2). The GSTP1 is involved, among other GST, in different cancer susceptibilities. The variant GSTP1 polymorphism Ile105Val may modulate the colorectal risk (58). A better survival rate was obtained not only for patients with 105Val homozygote alleles with colorectal cancer (59), but also for breast cancer (60), head and neck cancer (61) treated with chemotherapy. The mutated variant of GSTP1 appears if there is a single nucleotide substitution (A→G) at position 313 of the GSTP1 gene, which will produce a substitution of Ile (isoleucine) with Val (valine) at codon 105, and a decrease of the catalytic activity of GSTP1, especially if both alleles are modified, an intermediate activity of the protein being present when only one allele is modified (the patient is heterozygote).

We do not have the chemotherapy protocol followed by our colorectal patients and we also cannot make assessments about its efficiency. Our study consists of a small number of patients collected in more than 7 years from a larger number of patients

having colorectal cancer. The death rate was due to a stage IV cancer disease. In cases where a total colonoscopy is impossible to make because of an obstruction and presentation in emergency, we need to perform the entire colonoscopy after surgery due to possible synchronous malignant lesions which cannot be intraoperatively discovered by manual palpation (62). An aggressive surgical approach means performing a total colectomy with an ileo-rectal anastomosis (63) and, where polyps or other lesions in the rectum are present, a rectocolectomy or even a proctorectocolectomy with ileostomy could be performed.

The risk for colorectal cancer was increased for GSTM1 and GSTT1 null genotypes and also for the association of all three GST-ies, but not for GSTP1 Ile 105Val polymorphism (56), confirmed also in a large meta-analysis (64) for the Caucasian, but not the Chinese population. We found that GSTM1 null genotype in our patients is a significant risk factor for colo-rectal synchronous cancers and also for a synchronous association of colorectal and other types of cancer. We did not find significant association with null GSTT1 genotype or GSTP1 Ile105Val polymorphism. This could be explained by the small number of cases, by the fact that a great proportion of the Caucasian population has a GSTM1 and GSTT1 null genotype and that the controls were selected from the general population. Another explanation may be that in Romanian people the other GST-ies polymorphisms are not strongly linked with an increased risk for colorectal multiple cancers.

Conclusions

Clinical surveillance of the patients diagnosed with a colorectal cancer is of crucial importance, especially of those with multiple colorectal cancers, synchronous or metachronous. We do not know yet all the genetic alterations which could lead to tumor progression, but multiple cancer syndromes have been and continue to be studied, and as time will pass more other genetic changes will be discovered. GST-ies were largely studied in the last years and it was discovered that they are linked to many different cancers, not only colorectal cancers. Their proteins have a very important role in cell defence, being capable to take out from the cell many carcinogen agents. We observed that the null GSTM1 genotype is a risk factor for synchronous multiple colorectal cancers and colorectal cancers associated with other cancers.

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